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The Nutritional Adequacy of Five Strains of Bacteria for Vorticella microstoma and Oxytricha sp.

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The Nutritional Adequacy of Five Strains of Bacteria for *Vorticella microstoma* and *Oxytricha* sp.

(TITLE)

BY

Michael J. Sinsko

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THESIS

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Presented by

Michael J. Sinsko

a candidate for the degree of Master of Science

and hereby certify that in their opinion it is acceptable.

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LITERATURE REVIEW

The disposal of human and industrial waste products and the impact of these substances on the natural environment had been cause for concern at the time of Marsson (1911) who, though realizing the existence of a problem, still considered a little bit of pollution a good thing. With the growth of population centers the waste-treatment-pollution dilemma increased, providing incentive for the study of methods for the analysis and improvement of the quality of sewage treatment procedures. Lackey's (1949) contention that because a biological process is required for complete treatment of almost all sewage, the specific details concerning its action should be known, became more apparent.

Butterfield (1928) attempted to determine why there is a tendency toward a consistent and rapid decrease in numbers of bacteria in sewage-polluted streams, though the same waters, collected in artificial containers and observed in the laboratory, exhibited an increase in numbers. The observation of Swaminathan (1929) that Vorticella in activated sludge used bacteria as a food supply, followed by Barritt's (1940) observation that properties of activated sludge are dependent upon definite groups of organisms which appeared to be mutually selective, preceded studies of the relationship between effluent quality and protozoan fauna.

The importance of the relationship between the quality of sewage effluent and the number of Vorticellidae was recognized by Reynoldson (1942) and Pillai and Subrahmanyan (1942). Barber (1942) assessed the role of Protozoa in the Bacteria Bed Process of sewage purification

compared with that in Imhoff digestion tanks. Further studies by Pillai and Subrahmanyan (1944) and Allen (1948) emphasized the importance of Protozoa in the purification of sewage. Speculation concerning the mechanism of purification was strongly influenced by the fact that Pillai (1941, 1942) and Pillai, Wadhwani, Gurbaxani and Subrahmanyan (1947) found Vorticella and Epistylis to be more efficient in the flocculation of organic matter in sewage than bacteria. Flocculation of bacteria by protozoans was later studied by Hardin (1943), Watson (1945), and Curds (1963). The importance of members of the Vorticellidae and Epistylidae to formation of healthy, active sludge was emphasized by Pillai and Subrahmanyan (1943) who found that the bulking of sludge is traceable to the destruction of these protozoans. The results of similar work by Jenkins (1942), however, led him to state that there was not enough supporting evidence to make "sweeping generalizations" about the importance of Protozoa in activated sludge.

Meanwhile, Pillai, Wadhwani, and Subrahmanyan (1947) discovered that the respiration rate of Vorticella is higher than other protozoans in sewage purification, which might be linked with Allen's (1944) attempt to establish a relationship between aeration of sewage and numbers of bacteria. Lackey (1932) had discussed the need for aeration by certain protozoans in sewage treatment plants.

Protozoan distribution and the factors influencing it has been discussed by several authors. According to Curds (1966), Buswell and Lang were the first to indicate a succession of protozoans during the maturation of sludge. The results of the study of Noland (1925), which indicated that the most important factor in the distribution of freshwater ciliates was the amount of available food, was confirmed by the

work of Lackey (1938) who also found that though a protozoan species may be cosmopolitan, there is little probability of finding any given species in a chance habitat. Cutler, Crump, and Dixon (1932), who studied factors affecting Protozoa in biological filters and found that the food supply influenced distribution, theorized that when chemical compounds added to solution affect protozoan populations adversely, it may be due to the development of a bacterial flora detrimental to the Protozoa.

Further investigations on the succession of protozoans in sewage treatment was done by Baines, Hawkes, Hewitt, and Jenkins (1953) who studied relationships between biochemical oxygen demand, dissolved oxygen, and populations of ciliates, finding an inverse correlation between numbers of peritrichous ciliates in activated sludge and the BOD of the effluent. McKinney and Gram (1956) tried to correlate the work of previous investigators on the role of Protozoa in activated sludge with the concepts of competition and domination among species, and Stout (1956) attempted to relate ciliate ecology to their response to specific environmental factors by reviewing the work done by previous authors.

In a study of protozoan successions in a diffused air-activated sludge plant, Brown (1965) found drastic changes in the environment did not disrupt regular patterns of life and development. Curds (1966) suggested reasons for the sequence of ciliates and limitations of their use as indicator species and reaffirmed the importance of ciliates to effluent quality in sewage treatment plants (Curds, Cockburn and Vandyke, 1968). Noland and Godjics (1967) summarized much of the work which had been done to determine environmental factors influencing protozoan distribution. Reid (1969) studied the relationships of populations of three species of Vorticella to varied bacterial activity in activated sludge plants.

Evans and Beuscher (1970), in their study of the succession of protozoans in tertiary sewage treatment, noted the role of ciliates in the clearing of the effluent.

In order to effectively study the relation between the bacterial-feeders of the protozoan fauna and the bacterial flora, research had to be done on the nutritional requirements of ciliates. Robertson (1921) put forth the theory of autocatalysis, involving a certain "X"- substance, to explain the accelerated rate of division through the mutual proximity of two protozoans in the presence of a minimum amount of food. Cutler and Crump (1923a, 1923b, 1924), Hetherington (1934), Burbanck (1942), and Burbanck and Gilpin (1946), studied the effect of various bacteria on the growth and division rate of Colpidium colpoda. During the process of this research, Cutler and Crump (1923b) tested Robertson's theory of autocatalysis but were unable to find evidence of its existence. Robertson (1924) tried to explain the reason for the failure of these authors to observe the effect. Cutler and Crump (1924) were able to show, however, that the number of divisions steadily decreases as the number of inoculated animals increases. Later, Johnson (1933), in his study of the effects of population density on the rate of reproduction of Oxytricha observed that Robertson's results can be explained on the basis of the ratio between the ciliate and bacterial populations.

Kidder and Stuart (1939a), studying the effect of various strains of bacteria on the growth and reproduction of Colpoda, found that some bacteria, in appreciable concentrations, may be detrimental to ciliates. They had earlier found that Flavobacterium was non-toxic, but was rejected as food by ciliates, and that Chromobacterium violaceum caused death even in small concentrations (Kidder and Stuart, 1938). Subsequent study using

Aerobacter suspended in distilled water showed that products of ciliate disintegration can be toxic to ciliates, also causing very rapid agglutination of bacteria (Kidder and Stuart, 1939b). Burbanck and Gilpin (1946) discussed the possibility of using the division rate of Colpidium colpoda as an aid to taxonomic studies of bacteria and the diagnosis of pathogenic infections. Curds and Vandyke (1966) used strains of bacteria reported in sewage to determine feeding specificity of sewage-related protozoans. The culture media employed in these studies varied from synthetic medium plus a surplus of acceptable food (Cutler and Crump, 1923a, and Curds and Vandyke, 1966) to sterile artificial pond water (Burbanck, 1942). Levine (1960) cultured Vorticella convallaria using Cerophyl extract plus a suspension of coagulated egg yolk in distilled water.

Leslie (1940) attempted to qualitatively and quantitatively standardize the food supply of Paramecium multimicronucleata. Kidder (1941) worked on the nutritional requirements of four species of holotrichous ciliates. Previous studies performed to determine the factors controlling the growth of protozoan populations were reviewed by Johnson (1941).

The need to free ciliates from bacterial contaminants in order to obtain valid results was realized by several authors. Hetherington (1934) described a technique for sterilization of ciliates by combining washing and migration. Claff (1940) employed migration through sterile fluid in combination with a high dilution factor. He also summarized methods of sterilization employed to that point. Wagtendonk (1955) reviewed all work which had been done on the nutrition of ciliates, specifically with the requirements of ciliates in monoxenic and axenic culture.

A great deal of work on the peritrichous ciliates has been done by H. E. Finley and associates. Finley, McLaughlin, and Harrisson (1959) developed methods for the axenic and non-axenic growth of Vorticella microstoma. Finley and McLaughlin (1963) attempted to determine the nutritional and ecological requirements of peritrichs by sampling the physical conditions of natural habitats and relative populations of protozoans and bacteria over a period of time. Telotrochidium henneguyi was then cultured axenically in an unsuccessful attempt to determine its exact nutritional demands (Finley and McLaughlin, 1965). Culture techniques for some Peritrichida were discussed by Finley (1965) and summaries of all work done to date on the study and cultivation of peritrichs were made (Finley 1966, 1969). The importance of studies of nutritional requirements in cultures of Protozoa to the studies of a healthy activated sludge was discussed by Brown, Brown, and Reid (1965).

Studies on the conjugation, reproduction, encystment, growth, and sexual differentiation of ciliates, particularly peritrichs, were done by Finley (1936, 1943, 1952) and Finley and Lewis (1960).

Sampling techniques for sessile protozoans to aid in studies of polluted environments were described and developed by Sladeckova (1962), Spoon and Burbanck (1967), and Burbanck and Spoon (1967). These techniques employed the use of "aufwuchs" samplers.

Taxonomic work on sewage and pollution-related ciliates was done by Noland and Finley (1931), Reid (1967), Curds (1969), and Small and Ranganathan (1971).

The work of the authors cited has contributed toward a better understanding of the inter-relationships between protozoans and their environments, while providing a foundation upon which more work can be done.

INTRODUCTION

Several authors have attempted to specifically define the role of protozoans, particularly ciliates, in the purification process of sewage treatment and in the clearing of organically polluted streams. Results of these studies suggest that this role is intrinsically related to the quality of the environment in which these organisms are found. In the case of certain ciliates, this is a role which is determined by the bacterial flora of the surrounding medium.

The relationship between ciliate and bacterial populations in respect to the fulfillment of nutritional requirements of the ciliates is, therefore, of concern to those who wish to understand the mechanism by which the clearing of organic pollution occurs. To this end, several strains of bacteria have been isolated from Kickapoo Creek, an organically polluted stream in Coles County, Illinois, and used as food sources for cloned cultures of Vorticella microstoma and Oxytricha sp. which were isolated from the same habitat. The effect of each strain of bacteria upon these protozoans was then determined according to daily division rates.

METHODS AND MATERIALS

A point 0.74 miles downstream from the Mattoon, Illinois, Sewage Treatment Plant on Kickapoo Creek (Station I) was selected as a collection site from which protozoans and bacteria were to be isolated. The relative amount of pollution at the collection site was determined by a comparison between benthic organisms, biochemical oxygen demand, and dissolved oxygen levels at the collection site and two other points, 6.31 (Station II) and 10.03 (Station III) miles downstream. Benthic organisms were collected with a Serber's Square Foot Sampler and dissolved oxygen levels were determined through Winkler Analysis.

"Aufwuchs" samplers were used to collect sessile protozoans. These were made by removing the top and bottom of a plastic Carolina Biological Supply Co. slide box and inserting two slides, back-to-back in each one of six slots. The slides were held in place by rubber bands. Samplers were fastened one foot below the surface to stakes driven into the bottom of the creek at the collection site. After a period of two weeks, the slides were removed, examined, and protozoans adhering to the face of the slides were isolated and maintained in cultures containing 0.1% w/v Cerophyl extract and 0.01% w/v Difco coagulated egg yolk prepared according to the method described by Levine (1960). Aerobacter aerogenes was used as a food source.

Following a two week adjustment period, one individual from each culture of Vorticella microstoma and Oxytricha sp. was isolated. Clones

started from each of these were used for feeding experiments begun one month later.

Nineteen strains of bacteria were isolated by streaking creek water samples on plates of nutrient agar and isolating morphologically distinct colonies. The purity of these strains was then insured through a series of six sub-cultures using the streak method on nutrient agar. Slants were then prepared and kept refrigerated. Five strains were selected at random for feeding experiments.

Feeding experiments were conducted using modifications of the technique employed by Curds and Vandyke (1966). A single protozoan was isolated from cloned culture with a micropipette, and washed three times for five minutes each in sterilized culture medium. The protozoan was then placed in sterilized medium on a depression slide into which a small drop of bacterial growth from nutrient agar slants was suspended. The introduction of a small mass of bacterial cells insured an excess amount of food available over a twenty-four hour period.

The sterilized depression slide with medium, protozoans, and bacteria was placed on an angle of glass tubing set in the bottom of a sterilized petri dish and covered. Sterilized distilled water at the bottom of the petri dish prevented excess evaporation of the culture medium.

Twenty-four hours after inoculation, the number of protozoans was counted using the 40X scanning power of a compound microscope for the Vorticella and a 20-60X dissecting scope for the Oxytricha. Six individuals of each of the two genera of Protozoa were isolated, washed three times, and two were placed in each of three depression slides. The total number of animals was again counted after twenty-four hours and 6 were

isolated from one depression slide selected at random, followed by the same washing procedure. Protozoan counts and transfers were made daily for 10 successive days, or until all Protozoa died. The daily division rate (R) was then calculated according to the formula:

$$R = \frac{\log B - \log A}{\log 2}$$

(A) equals the number of protozoans on day 1 and (B) equals the number on day 2 (Curds and Vandyke, 1966).

Once during each 10-day series a loop-full of culture medium with food bacteria was streaked onto a plate of nutrient agar after the bacteria and protozoans had been in the medium for twenty-four hours. This procedure served to check for contaminants and to insure that viable food bacteria still remained after a twenty-four hour period.

According to their relative performances, each of the five strains of bacteria were classified as Toxic, Nutritionally Inadequate, or Nutritionally Adequate. Toxic bacteria are those bacteria in the presence of which ciliates will not remain alive for at least twenty-four hours. Bacteria which will not support reproduction for at least eight days were classified as Nutritionally Inadequate. Nutritionally Adequate bacteria are those strains with which reproduction continued to occur through the end of the tenth day.

All operations were performed beneath a hood on a sterilized piece of satin cloth. Autoclaved micropipettes, beakers, depression slides, and petri dishes were used for all operations.

Bacteria were identified according to Breed, Murray and Smith (1957) and Skerman (1967). The protozoans were identified according to descriptions in Kudo (1966) and Curds (1969).

OBSERVATIONS AND RESULTS

The relative abundance of pollution-associated benthic organisms such as the larvae of Tendipedidae and Tubificidae was larger at Station I (the collection site) than at Stations II and III (Table 1). Water quality tests (Tables 2 and 3) also indicated a higher degree of organic pollution at Station I than at the other two stations.

Isolated bacteria strains which were selected for feeding experiments were identified as Chromobacterium violaceum, Brevibacterium lipolyticum, Kurthia zopfii, Proteus rettgeri, and Flavobacterium sp. Flavobacterium sp. was identified only to genus since its physiological characteristics did not appear to match any of the species descriptions in Breed, Murray and Smith (1957).

According to their suitability as food organisms, as determined through the continued reproduction of Vorticella microstoma and Oxytricha sp. (Table 4), each of the bacterial strains was placed into one of three categories. These categories: Nutritionally Adequate, Nutritionally Inadequate, and Toxic are based on the length of time during which the protozoans were able to be maintained. Thus, Chromobacterium violaceum is toxic to both Vorticella microstoma and Oxytricha sp. (Table 4), Flavobacterium sp. is Nutritionally Inadequate for Vorticella microstoma and Toxic for Oxytricha sp. (Fig. 5), Brevibacterium lipolyticum is Nutritionally Inadequate for both Vorticella microstoma and Oxytricha sp.(Fig. 4), Proteus rettgeri and Kurthia zopfii (Figs. 2 and 3) are Nutritionally Adequate for both Vorticella microstoma and Oxytricha sp. The latter two

bacterial strains are in varying degrees of adequacy as compared to the reaction with Aerobacter aerogenes (Fig. 1) which is used as a point of reference.

What appeared to be conjugation was observed twice during feeding experiments with Vorticella microstoma. This phenomenon was not observed with Oxytricha sp.

TABLE 1

BENTHIC ORGANISMS COLLECTED IN SERBER'S SQUARE FOOT SAMPLER. TEN SITES SAMPLED AT EACH OF THREE STATIONS.

STATION	TUBIFICIDAE	TENDIPEDIDAE LARVAE
I	300+	83
II	24	20
III	0	55

TABLE 2

RESULTS OF BIOCHEMICAL OXYGEN DEMAND (mg/l) DETERMINATIONS TAKEN AT EACH OF THREE STATIONS ON KICKAPOO CREEK AT 2-WEEK INTERVALS.

SAMPLE NO.	STATION I	STATION II	STATION III
I	12.0	4.0	4.0
II	9.0	1.0	0.8
III	14.3	4.4	6.6
IV	4.0	2.2	0.4

TABLE 3

RESULTS OF DISSOLVED OXYGEN (mg/l) DETERMINATIONS TAKEN AT EACH OF THREE STATIONS ON KICKAPOO CREEK AT 1-WEEK INTERVALS.

SAMPLE NO.	STATION I	STATION II	STATION III
I	8.0	9.1	9.5
II	6.4	8.8	9.1
III	6.4	8.6	8.6
IV	5.6	7.0	7.6
V	6.3	6.9	7.1
VI	4.8	6.4	6.6
VII	3.6	6.1	7.3

TABLE 4

MEAN DAILY DIVISION RATES (N=3) OF *VORTICELLA MICROSTOMA* AND *OXYTRICHA* SP. AFTER FEEDING WITH STRAINS OF BACTERIA ISOLATED FROM KICKAPOO CREEK.

Protozoan and Bacterial Food Source		Daily Division Rate									
		1	2	3	4	5	6	7	8	9	10
Vorticella microstoma	*Chromobacterium violaceum*	0									
	Flavobacterium sp.	1.67	0.14	0.33	0						
	Brevibacterium lipolyticum	0.67	0.20	0							
	Kurthia zopfii	2.83	2.29	1.27	0.21	1.50	3.60	0.87	0.49	0.49	0.74
	Proteus rettgeri	0.67	2.61	0.67	1.97	2.62	0.94	1.27	1.74	2.02	1.34
	Aerobacter aerogenes (Control)	1.78	1.94	2.07	1.77	2.19	1.88	0.22	0*	0.74	4.62
Oxytricha sp.	*Chromobacterium violaceum*	0									
	Flavobacterium sp.	0									
	Brevibacterium lipolyticum	0.77	0.23	0.67	0.67	1.94	0.29	0*	0		
	Kurthia zopfii	1.16	1.43	0.79	1.35	1.25	2.58	0.42	0.55	0*	0*
	Proteus rettgeri	1.00	1.16	1.00	1.16	0.96	1.35	1.21	0.55	0.30	0.94
	Aerobacter aerogenes (Control)	1.73	1.12	1.32	3.17	2.56	2.47	0*	3.61	2.32	1.41

*Indicates viable organisms still remain though division rate is 0.

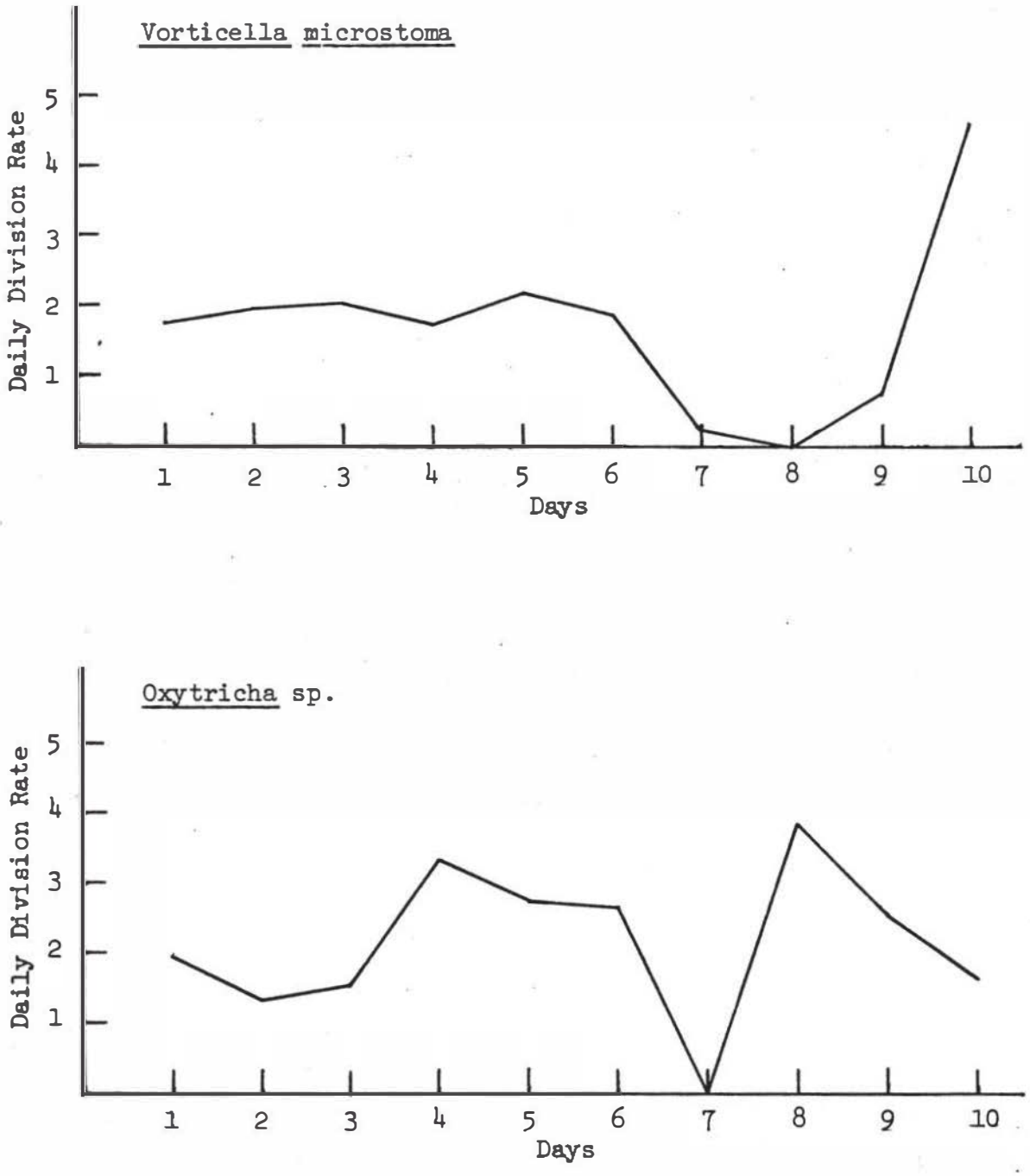

Fig. 1. Mean Daily Division Rates of Vorticella microstoma and Oxytricha sp. when fed with Aerobacter aerogenes.

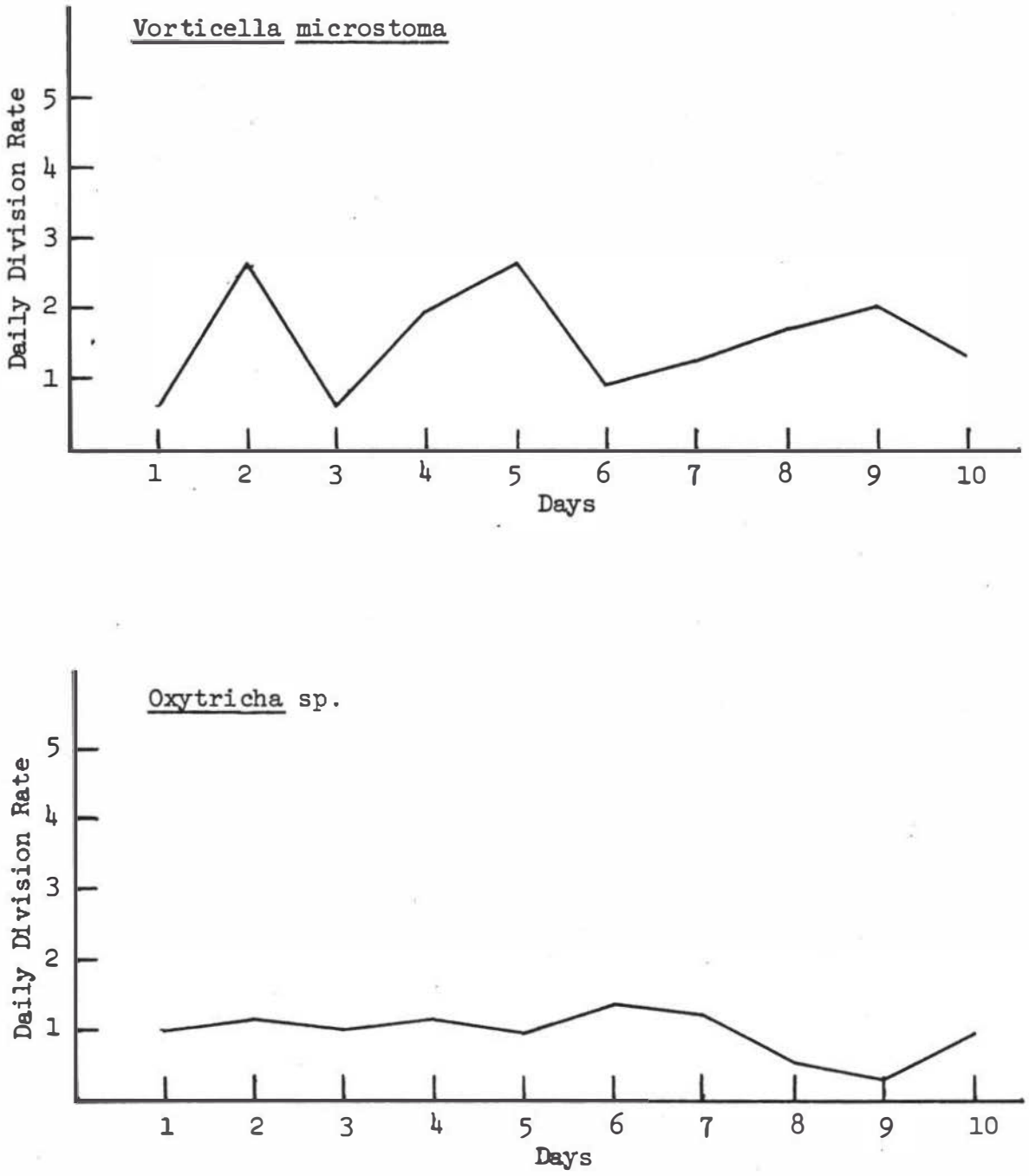

Fig. 2. Mean Daily Division Rates of Vorticella microstoma and Oxytricha sp. when fed with Proteus rettgeri.

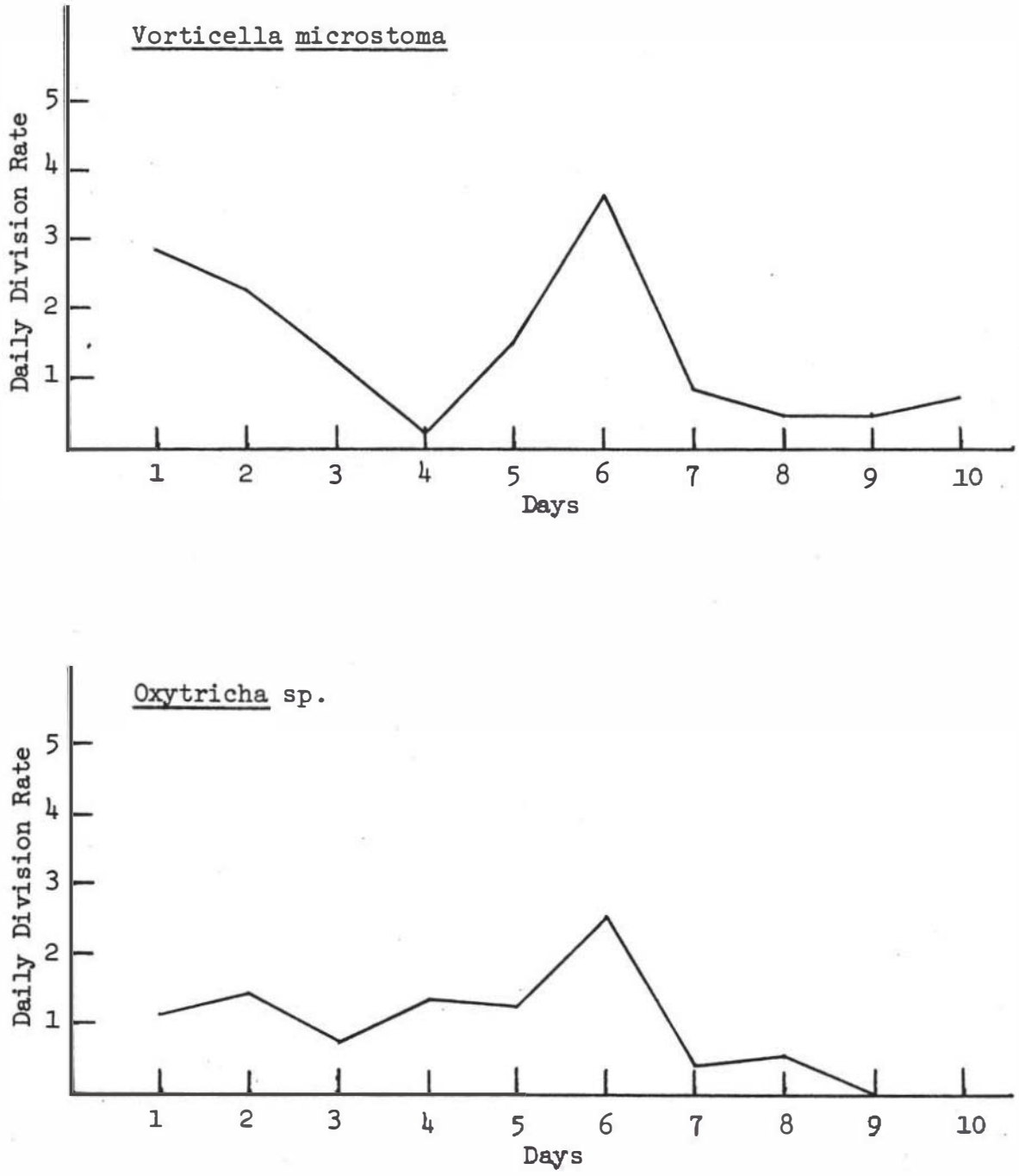

Fig. 3. Mean Daily Division Rates of Vorticella microstoma and Oxytricha sp. when fed with Kurthia zopfii.

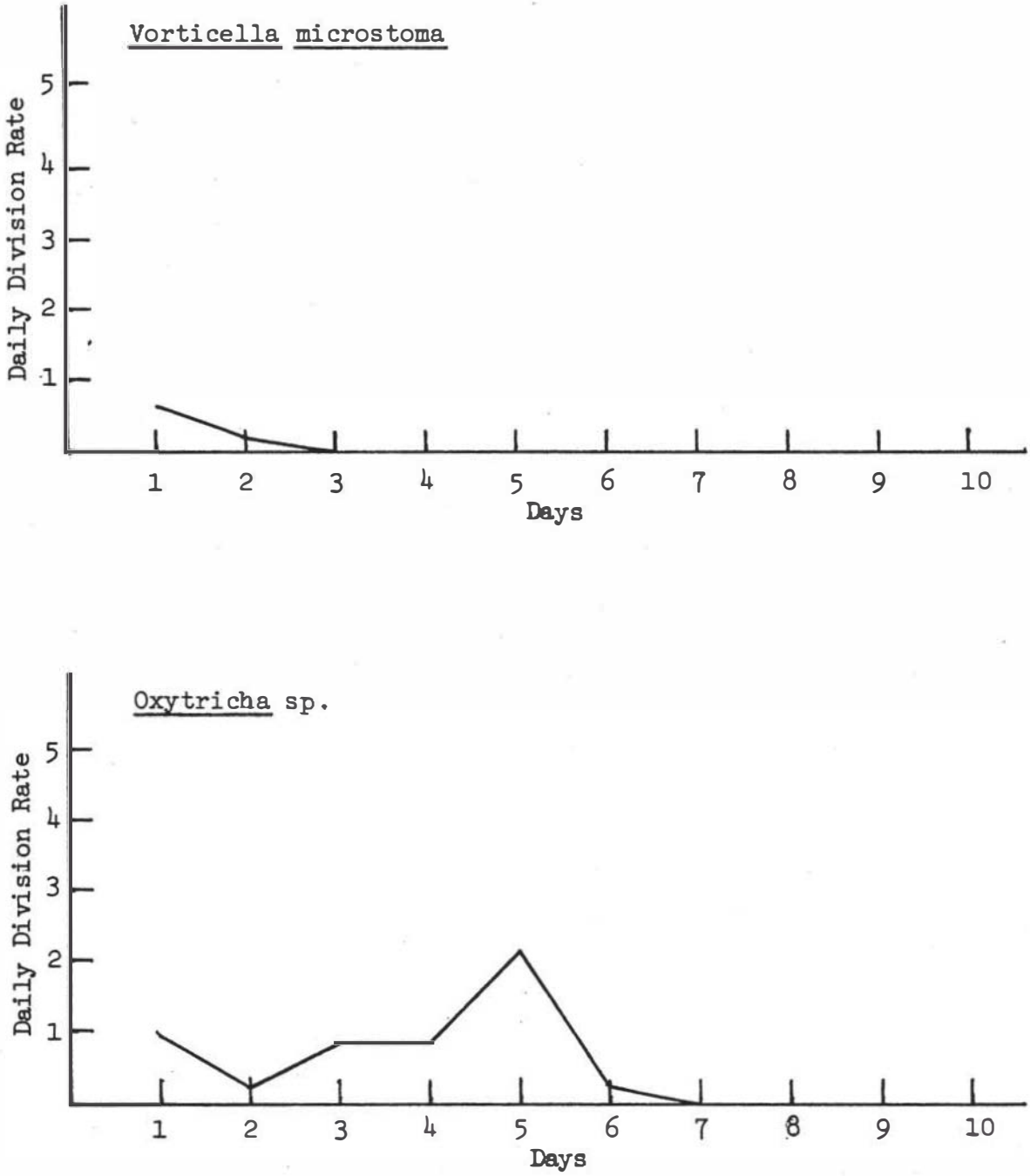

Fig. 4. Mean Daily Division Rates of Vorticella microstoma and Oxytricha sp. when fed with Brevibacterium lipolyticum.

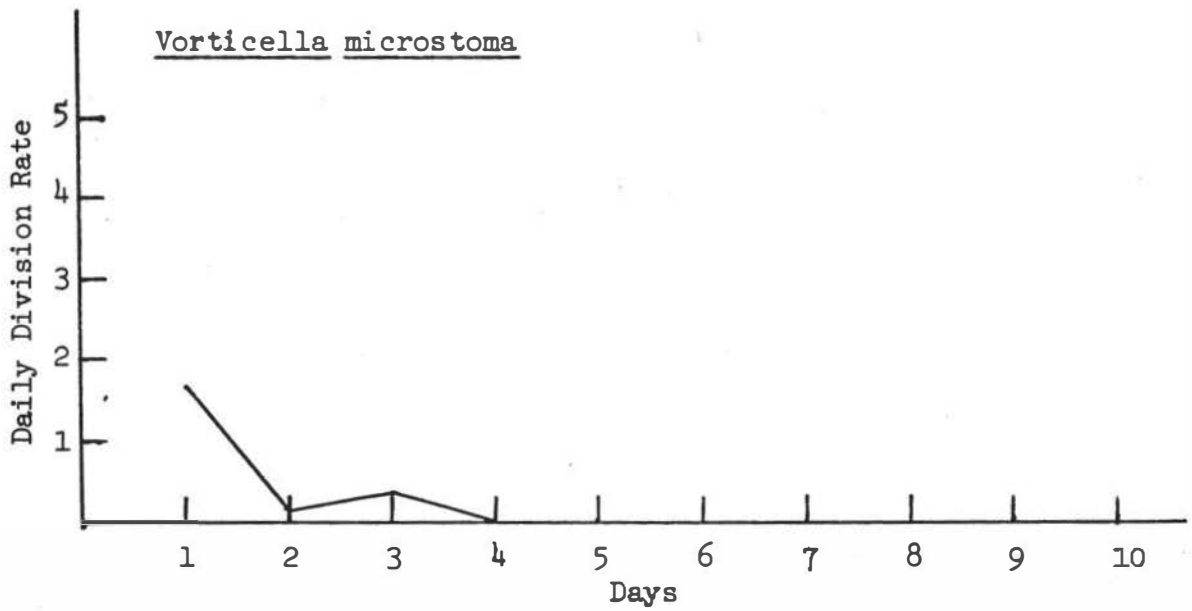

Fig. 5. Mean Daily Division Rate of Vorticella microstoma when fed with Flavobacterium sp.

DISCUSSION

Because of the nature of this investigation certain techniques used by other workers have had to be modified.

"Aufwuchs" samplers were employed to obtain protozoans in order to insure that those isolated would be indigenous to a particular habitat at the time of collection. In a moving body of water, at a site relatively close to the point of discharge of a qualitatively uncontrollable effluent, there would be no guarantee of a stabilization of physical and biological factors for any length of time. Such conditions would affect a population of sessile peritrichs such as Vorticella which have the capacity to quickly form telotroch stages in response to adverse environmental changes and move to a more suitable location.

However, though the potential for radical changes in environmental conditions exists, the numbers of pollution-associated organisms collected at the three stations, such as Tubificidae and Tendipedidae larvae, indicate a relatively high degree of organic pollution at Station I in comparison with the other two stations downstream (Table 1). This condition must be relatively static in order to allow populations of these organisms to become established in numbers as great as that which had been found. The results of the series of Dissolved Oxygen and 5-day Biochemical Oxygen Demand determinations which were performed at one and two-week intervals over a 2-month period of time confirmed this (Tables 2 and 3).

The isolation culture technique described by Curds and Vandyke (1966) was tried as a means of determining relative rates of reproduction

influenced by various bacterial food sources. These authors prepared isolated compartments by affixing glass rings into molten agar in petri dishes which were then autoclaved. These were used for free-swimming ciliates. Peritrichs were cultured in depression slides into which a drop of molten agar had been placed, followed by sterilization in a moist chamber. In both instances, the bacteria to be used as a food source was streaked onto the agar within the chamber.

A few difficulties, however, were presented by the use of this method. First, bacterial growth in the glass rings extended beyond the confines of the ring. Second, diffraction of light through the agar made observation of protozoans within the rings extremely difficult, hindering attempts to make accurate counts. Third, in both the rings and in the depressions, the growth of bacteria concentrated on the agar at the bottom of the chambers was so dense as to obscure the view of the protozoans. Fourth, since the glass sides of the chamber afforded a more secure site of attachment for the Vorticella than the agar, this is where most of them settled. This made prying them loose for transfer, without injury, extremely difficult. Following a few trials, it became apparent that certain modifications were necessary.

A method was devised in which bacterial cells were suspended in sterile culture medium in a depression slide. As long as the bacteria introduced as a food source could remain viable for the twenty-four hour study period in the sterilized medium, this was perfectly adequate. The ability of the bacteria to remain viable was assured by streaking a loop-full of the medium on a nutrient agar plate prior to transfer. This also aided in the detection of contaminants. Since the colonies formed by the strains of bacteria used as food sources differed morphologically from

each other, as well as from Aerobacter aerogenes when cultured on nutrient agar plates, this expedited detection without microscopic examination.

The validity of the triplicate isolation technique was statistically tested by Curds and Vandyke (1966). The test showed that the mean division rates of the three possible lines originating with a single individual, if followed through, are not significantly different. One line, selected at random, could therefore be followed.

The selection of an adequate culture medium, using Aerobacter aerogenes as a bacterial food source, was made after experimentation. Ideally, the water from which the organisms were isolated should have been used in the culture medium. However, the use of Kickapoo Creek water was decided against. Water from the creek, after autoclaving, was tried first with Aerobacter aerogenes, second in combination with dessicated lettuce leaves and coagulated egg yolk as described by Levine (1959), and third with Cerophyl and coagulated egg yolk (Levine, 1960). However, the best growth of Vorticella microstoma, in culture, was obtained using a medium composed of Cerophyl extract and coagulated egg yolk in distilled water. Because of the results of these tests, and the possibility of fluctuations in creek water quality, the latter medium, which can be reproduced exactly, was used. Though Burbanck (1942) used sterile artificial pond water in division rate studies of Colpidium colpoda, the advantage of using an easily reproduceable medium is discussed by Cutler and Crump (1923a).

As will be discussed later, Aerobacter aerogenes, though suitable for maintenance of cloned cultures, was found to be less than ideal as a food source for Vorticella microstoma. This became evident from observations of the Vorticella microstoma and Oxytricha sp. cultures kept in

Syracuse Watch Glasses with approximately 10 ml of Cerophyl-coagulated egg yolk medium. If the medium was not changed completely at least every fourth day, the Vorticella showed signs of deformation, becoming smaller and appearing to shrivel, even though fresh bacteria cells were added daily. The Oxytricha sp., however, were able to remain in the same medium without any apparent deformation, though they did get progressively smaller after a period of time.

These effects may be attributed to an accumulation of metabolic wastes, lack of aeration, inadequate nutrition, or a combination of these factors. Analyzing each of these elements individually, aeration, in the case of Vorticella, is important as has been shown by Pillai, Wadhwani, and Subrahmanyan (1947). Within such a short period of time, however, aeration should not have been a major factor since a small amount of culture medium with a relatively large amount of exposed surface area was used. Metabolic wastes in a quantity of medium (in the watch glasses) twenty times greater than that in the depression slides should not have accumulated in only four times the amount of time (4 days) to the extent that deformities in morphological integrity took place and reproduction completely stopped. The toxicity of ciliate disintegration products as hypothesized by Kidder and Stuart (1939b) might be considered but for the fact that they had used extremely small volumes of medium (0.005 ml) and an excess of Aerobacter aerogenes with a resultant 20,000 ciliates after 48 hours. Also, although according to Hetherington (1933), it was discovered by Luck, Sheets, and Thomas that after long time-spans ciliate cultures may die out, senescence would hardlybe probably in so new a culture. The most logical explanation, therefore, would be nutritional deficiency. However, the key factor seems to be the culture medium since an excess of bacterial cells were readily available.

So little is known about ciliate nutritional requirements that only a certain amount of conjecture is possible. The dearth of knowledge in this area of Protozoology was emphasized by a fact made known by Holz (1964) who stated that of 6,000 species of ciliates described by Corliss in 1961, only two species had been available, as of 1958, in a chemically defined medium.

Since Vorticella microstoma, upon trial, could not be maintained on Aerobacter aerogenes and distilled water alone, it is evident that a certain amount of nutritional material from the culture medium is utilized. The culture medium, containing Cerophyl extract (a vitamin supplement from cereal grass leaves) and coagulated egg yolk, was autoclaved before use, a process which would be expected to denature the vitamins. The Cerophyl, however, still must have provided a necessary constituent since a medium composed of coagulated egg yolk extract alone would not support a culture of Vorticella microstoma, even with Aerobacter aerogenes. There exists, therefore, the possibility that following a relatively short period of time, the substance in the medium providing the nutritional factor which the bacteria alone cannot provide, is used, dissipated, or in some manner lost.

Regardless of the apparent insufficiency of Aerobacter aerogenes as a sole food source, this species of bacteria was used as a control. Knowing that it does have the ability to maintain cultures, however minimally, it can be used as a point of reference in the comparison of division rates.

Chromobacterium violaceum had been found to be toxic to ciliates by Kidder and Stuart (1938 and 1939a), Burbanck (1942), and Curds and Vandyke (1966). This was found to be true in this study for both Vorti-

cella microstoma and Oxytricha sp. The cause of this toxicity was attributed by Curds and Vandyke (1966) to be the pigment "violacein" which is produced by the bacteria.

Similarly, Kidder and Stuart (1938 and 1939b) found Flavobacterium to be non-toxic though rejected as a food source by ciliates. This was supported by the results with Vorticella microstoma which continued to reproduce for only three days at a very minimal rate (Fig. 5). The continuation of reproduction may, however, be due to nutrient reserves in food vacuoles previously formed while the organisms were in culture with Aerobacter aerogenes, or to such bacteria cells caught in the buccal cavity and not eliminated through washing. On the other hand, Flavobacterium appeared to have a toxic effect upon Oxytricha with no viable organisms present after twenty-four hours. Flavobacterium was, therefore, classified as Toxic to Oxytricha sp. and Nutritionally Inadequate for Vorticella microstoma.

Brevibacterium lipolyticum was classified as Nutritionally Inadequate for both Vorticella microstoma and Oxytricha sp., though there is a great difference in degree of insufficiency. Vorticella microstoma continued to reproduce very weakly for two days, indicating, once again, the possibility of responsibility for short-term continuation of reproduction to remnants of Aerobacter aerogenes from the original culture. Oxytricha sp., however, continued to reproduce for six days, with all organisms finally dying off on the seventh day. Here, the arbitrary limit of eight days, which had been set to separate categories of adequacy, based on the classification used by Curds and Vandyke (1966), may be questionable.

Both Proteus rettgeri and Kurthia zopfii supported reproduction in the Vorticella microstoma series through the tenth day, though Proteus rettgeri did so at a higher rate (Table 3). Oxytricha sp. continued to reproduce through the tenth day with Proteus rettgeri, but failed to do so past the eighth day with Kurthia zopfii. In the latter case, care must be taken in the interpretation of results since, though no reproduction took place, viable organisms were still present through the tenth day. The potential for reproduction past this point, therefore, still remained. Taking this factor into consideration, Kurthia zopfii should still be classified as Nutritionally Adequate for Oxytricha sp.

As can be seen in Figure 1, though Aerobacter aerogenes supported reproduction through the tenth day, the reproduction rates of Vorticella microstoma and Oxytricha sp. fell to zero at one point during the series. The reason for this drop is unknown.

Though precautions had been taken to reduce variables, there exists the possibility of factors such as aging of the bacterial cultures and protozoan clones with the accompanying possibility of conjugation which are beyond the control of the investigator. Cloned cultures of ciliates had been used to reduce the influence of any genetically controlled differences in feeding behavior. According to Kudo (1966) Jennings found that since ciliates in a clone are of the same mating type, they will not conjugate except after long periods of time (2,000 culture days). After this time there may be a transformation to two mating types, making conjugation possible. Though conjugation was induced by Finley (1936) in cultures of Vorticella only following excystment, it is significant that what appeared to be conjugation was observed twice in this study

during feeding experiments with Vorticella microstoma. The implication of this unique observation, if accurate, involves a possible reduction of genetic homogeneity due to gene recombination during conjugation. This was not observed, however, in the cultures of Oxytricha sp.

Conjugation in the cultures of Vorticella microstoma may also influence daily division rates since according to Finley (1943), eighteen to twenty-four hours are required for nuclear reorganization following conjugation. During this period of time, therefore, no division should be expected to occur.

It is also interesting to note that in the use of the triplicate isolation technique, two ciliates are usually used in one depression at the start of each twenty-four hour period (Curds and Vandyke, 1966). This was probably done in order to increase the chances of obtaining readable results, since the death of only one individual following transfer, regardless of the cause of mortality, would bring an end to the series. It was found, however, that in those series which were started with two ciliates, the average daily division rate was 1.16 while those started with a single individual had an average division rate of 1.56. This observation, though the difference was not large, might lead one to question the need to employ two ciliates to start each series instead of only one.

Another possible variable is the aging of cultures of protozoans and bacteria as feeding experiments are being carried out. The significance of aging of a cloned culture of ciliates was discussed in connection with conjugation. Bacteria, as in the case of some strains of Pseudomonas which lose the ability to produce pigmentation after a

period of time in artificial culture, may change characteristics which would affect their degree of adequacy as a food source. These difficulties, however, are hypothetical variables, the degree of which, at this time, cannot be determined, and as such need only be kept in mind while viewing the results.

While practical application of the results of studies such as this must necessarily wait until more work is done and information accumulated, a number of inferences can be drawn. The main impact would most likely be in attempts to control organic pollution of waterways through the improvement of sewage effluent quality. It is probable that since sewage treatment in Mattoon consists of only a trickling filter, further breakdown of organic wastes, plus the clearing of the products of this breakdown, must occur in Kickapoo Creek. The role of ciliates in the flocculation of bacteria and organic debris in tertiary sewage treatment plants indicates the probability of similar activity occurring within the creek. The mechanism of this flocculation has been the source of increasing interest as the forementioned role of ciliates in the clearing of organic pollution becomes more apparent.

Flocculation, as demonstrated with the use of India ink particles by Paramecium caudatum, has been ascribed by Curds (1963) as the result of a dual mechanism: through the secretion of a polysaccharide substance into the surrounding medium and through ingestion of particles by the cell followed by release with a mucoprotein bond. Watson (1945) found similar results with Balantiophorus minutus, in which flocculation of bacteria by a "mucus" preceded ingestion, apparently aiding the feeding process. Curds and Vandyke (1966) suggest that the formation of empty

food vacuoles seen in their studies are indicative of selection on the part of ciliates, since bacteria had been observed being rejected by Paramecium caudatum under phase contrast microscopy.

The assumption that a certain amount of selectivity on the part of the ciliates occurs, though very possibly true, is one which is made rather hastily based upon evidence gathered thus far. The very fact that Paramecium caudatum ingested non-nutritive India ink particles, while flocculation occurred regardless of this fact, suggests that in nature this mechanism may very well function haphazardly in the presence of suspended particles, regardless of their value to the flocculating organism. It is entirely possible that feeding may be aided only incicentally as a result of this flocculation, which may explain the empty food vacuoles and rejection of individual bacterial cells.

The answer to this question might be demonstrated rather simply once feeding experiments are perfected to the point where the exact reaction of ciliates when exposed to bacterial cells of varying degrees of nutritional adequacy can be determined. When this is established, the exposure of these protozoans to a mixture of two strains of bacteria, one nutritionally adequate and the other nutritionally inadequate, should yield the division rate normally expected with the nutritionally adequate strain if selection is taking place. A lowering of this rate, on the other hand, might indicate a haphazard ingestion of particles at a normal rate, but with ingestion of varying proportions of nutritionally adequate cells to those that are inadequate.

Whether the ingestion of particles for feeding or as a mechanism for flocculation is haphazard or selective, the mechanism of toxicity in certain bacteria is a critical factor. If the ingestion of toxic

bacterial cells necessarily precludes mortality, the presence of these cells would not affect protozoans if ingestion is selective. The results of secretion of toxic substance by the bacteria, however, would not be affected by selective or haphazard feeding. In the case of Chromobacterium violaceum, the production of a pigment seems to be fairly well incriminated. Whether or not this was the reason for mortality in Oxytricha sp. when exposed to Flavobacterium sp., especially since the Vorticella microstoma did not react as if exposed to a toxic substance, is an interesting question. The control of bacterial flora which would be nutritionally adequate for the maintenance of a protozoan fauna capable of efficiently flocculating out unwanted particles suspended in a body of water would be of prime importance in the biological control of organic pollution. The identification of nutritionally adequate bacteria would be a first step, followed by studies to determine whether or not large populations of these bacteria could be promulgated to the detriment of toxic and nutritionally inadequate forms without reducing the oxidation of organic wastes.

If feeding is selective, there would be an increase in the size of the protozoan population as a result of an increase in the proportion of beneficial bacterial cells. If feeding is haphazard, an increase in the proportion of beneficial cells would tend to give the protozoans a greater chance for ingestion of the beneficial forms. Flocculation, which is probably haphazard, would be increased through an expansion in the size of the ciliate population, raising the level of efficiency of the clearing machanism.

Control of protozoan fauna through a directed influence of bacterial flora is hypothetical and, if feasible, can be realized only after a con-

siderable amount of research. Hopefully, studies such as the one described in this paper will assist in the realization of this goal.

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